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(57) Abstract

Methods for inhibiting antigen-specific T cell responses *in vitro* by use of an agent which inhibits a costimulatory signal in T cells, such as a CTLA4Ig fusion protein or an anti-B7-1 antibody or an anti-B7-2 are disclosed. The methods of the invention are particularly useful for inhibiting graft versus host disease which results from allogeneic bone marrow transplantation.

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METHODS FOR INHIBITING GRAFT VERSUS HOST DISEASE IN BONE MARROW TRANSPLANTATION

Background of the Invention

Allogeneic bone marrow transplantation (BMT) is an effective treatment for many hematological malignancies and severe aplastic anemia (see e.g., Thomas, E.D. (1983) *J. Clin. Oncol.* 1:517-531; O'Reilly, R.J. et al. (1983) *Blood* 62:942-964; and Storb, T. et al. *Semin. Hematol.* 2:27-34). However, the alloreactivity of T cells within the donor bone marrow to recipient cells leads to a potentially fatal condition referred to as graft versus host disease (GVHD). One therapeutic approach which has been taken in an attempt to minimize or eliminate GVHD involves administration to the transplant recipient of a general immunosuppressant, such as cyclosporine A or methotrexate (see e.g., Kapoor, N. et al. (1989) *Bone Marrow Transplant.* 4:153). Use of such agents, however, is associated with deleterious side effects, including kidney damage and an increased susceptibility to infections. Another approach taken to minimize or eliminate GVHD has been to deplete donor bone marrow of T cells in an attempt to remove alloreactive T cells (see e.g., Martin, P.J. et al. (1987) *Adv. Immunol.* 40:379). While T cell depletion has been found to reduce the occurrence of GVHD, this treatment also reduces the success of bone marrow engraftment. Additionally, depletion of T cells from donor bone marrow used to treat hematological malignancies reduces the anti-leukemic activity (also referred to as the graft versus leukemia response, or GVL) of the donor cells (see e.g., Goldman, J.M. et al. (1988) *Ann. Intern. Med.* 108:806-814; Marmont, A.M. et al. (1991) *Blood* 78:2120-2130). Thus, while the presence of alloreactive T cells within a bone marrow graft has the detrimental effect of inducing GVHD, the presence of at least some T cells within the graft is beneficial both for successful engraftment and for anti-leukemic responses. A therapy that effectively inhibits the responses of alloreactive T cells within donor bone marrow while permitting the continued presence and function of other T cells within the graft would therefore be of great advantage in the addressing the problem of GVHD while promoting the efficacy of bone marrow engraftment.

The induction of a T cell response has been shown to require two signals: a first signal provided by stimulation through the antigen-specific T cell receptor (TCR) on the surface of the T cell, and a second signal (termed a costimulatory signal) provided by ligation of one or more other T cell surface receptors. Engagement of the TCR alone (i.e., signal 1) in the absence of a costimulatory signal (i.e., signal 2) induces a state of unresponsiveness, or anergy, in the T cell. A costimulatory signal can be generated in a T cell by stimulation of the T cell through a cell surface receptor CD28 (Harding, F. A. (1992) *Nature* 356:607-609). Ligands for CD28 have been identified on antigen presenting cells (APCs). CD28 ligands include members of the B7 family of proteins, such as B7-1(CD80) and B7-2 (CD86) (Freedman, A.S. et al. (1987) *J. Immunol.* 137:3260-3267; Freeman, G.J. et al. (1989) *J.*

Immunol. 143:2714-2722; Freeman, G.J. et al. (1991) *J. Exp. Med.* 174:625-631; Freeman, G.J. et al. (1993) *Science* 262:909-911; Azuma, M. et al. (1993) *Nature* 366:76-79; Freeman, G.J. et al. (1993) *J. Exp. Med.* 178:2185-2192). Additionally, B7 family members have been shown to bind another surface receptor on T cells related to CD28 termed CTLA4 (Linsley, P.S. (1991) *J. Exp. Med.* 174:561-569; Freeman, G.J. et al. (1993) *Science* 262:909-911).

The characterization of the receptors and ligands involved in T cell costimulation has led to therapeutic approaches based upon induction of antigen specific T cell unresponsiveness by blocking of a costimulatory signal in T cells. For example, a CTLA4Ig fusion protein, which binds both B7-1 and B7-2, has been used to inhibit rejection of cardiac allografts and pancreatic islet xenografts (see e.g., Turka, L.A. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89, 11102-11105; Lin, H. et al. (1993) *J. Exp. Med.* 178:1801-1806; Lenschow, D.J. et al. (1992) *Science* 257, 789-792). Similarly, antibodies reactive with B7-1 and/or B7-2 have been used to inhibit T cell proliferation and IL-2 production *in vitro* and inhibit primary immune responses to antigen *in vivo* (Hathcock K.S. et al. (1993) *Science* 262, 905-907; Azuma, M. et al. (1993) *Nature* 366:76-79; Powers, G.D. et al. (1994) *Cell. Immunol.* 153, 298-311; Chen C. et al. (1994) *J. Immunol.* 152, 2105-2114). However, effective methods for inhibiting T cell responses in bone marrow transplant situations which avoid the need for general immunosuppression of the transplant recipient and overcomes the drawbacks of T cell depletion in bone marrow transplants are still needed and would have widespread therapeutic applications.

Summary of the Invention

This invention features improved methods for inhibiting graft versus host disease in a bone marrow transplant recipient while preserving T cell mediated function against both tumor and pathogens in the recipient. This invention is based, at least in part, on the discovery that an inhibitor of a costimulatory signal in T cells can be used *in vitro* to inhibit inappropriate donor T cell responses to alloantigen and, thus, inhibit graft versus host disease in bone marrow transplantation. The inhibitor of a costimulatory signal in T cells is preferably an agent which inhibits an interaction between a costimulatory receptor on the T cell (e.g., CD28 and/or CTLA4) and a costimulatory molecule (e.g., B7-1 and/or B7-2) on a cell presenting antigen to the T cell. Thus, the inhibitor of a costimulatory signal can be, for example, an antibody (or fragment thereof) which binds the receptor or the costimulatory molecule, a soluble form of the receptor or costimulatory molecule or a peptide fragment or other small molecule designed to inhibit a costimulatory signal in T cells. A preferred inhibitor is a soluble CTLA4-immunoglobulin fusion protein (CTLA4Ig) or an anti-B7-1 antibody or an anti-B7-2 antibody. A much preferred inhibitor is a combination of an anti-B7-1 and an anti-B7-2 antibody.

According to the method of the invention, a T cell response is inhibited by contacting the T cell *in vitro* with at least one inhibitor of a costimulatory signal in an antigen specific T

cell. In particular, for use in inhibiting graft versus host disease in a bone marrow transplant recipient, an agent such as a combination of anti-B7-1 and anti-B7-2 antibodies or a soluble form of CTLA4 can be used to treat the donor bone marrow *in vitro*, to thereby inhibit donor T cell responses to cells expressing recipient alloantigens, prior to administration of the bone marrow to the recipient. For example, donor cells can be contacted with the inhibitor of a costimulatory signal in T cells *in vitro* in the presence of recipient cells and then administered to the recipient without further *in vivo* treatment of the recipient with the inhibitor. Alternatively, following administration of the treated donor cells, the inhibitor or other immunosuppressive agent (e.g., cyclosporine A) can be administered *in vivo* to the transplant recipient.

Brief Description of the Drawings

Figure 1 is a graphic representation of T cell proliferation in a primary mixed lymphocyte reaction between HLA disparate individuals treated with either CTLA4Ig, control immunoglobulin (Ig), or phosphate buffered saline (PBS) (control).

Figure 2 is a graphic representation of T cell proliferation in a primary mixed lymphocyte reaction between HLA disparate individuals treated with either anti-B7-1 monoclonal antibody, anti-B7-2 monoclonal antibody, both anti-B7-1 and anti-B7-2 monoclonal antibodies, CTLA4Ig, control immunoglobulin (CIg) or control antibody against peripheral blood lymphocytes.

Figures 3A and 3B are graphic representations of T cell proliferation in response to either NIH3T3 cells transfected with B7-1 (t/B7-1) or B7-2 (t/B7-2) or allogeneic peripheral blood lymphocytes bone marrow proliferation treated with various doses of CTLA4Ig (below 1 μg/ml:Figure 3A and from 1 to 10 μg/ml:Figure 3B).

Figure 4 is a graphic representation of the results of T cell proliferation assay of a secondary MLR against the original donor cells or third party cells following a primary MLR performed in the presence of media alone, cyclosporin A (CsA), an anti-MHC class II antibody, an anti-ICAM-1 antibody, CTLA4Ig, an anti-B7-1 antibody, an anti-B7-2 antibody, or a combination of an anti-B71 and an anti-B7-2 antibody.

Figure 5 a-d are graphic representations of the results of T cell proliferation assays of a secondary MLR after culturing the cells for 0, 0.5, 1, 1.5, 2, 3, 5, or 6 days in the presence of anti-B7-1 antibody (a), anti-B7-2 antibody (b), CTLA4Ig (c) or anti-B7-1 and anti-B7-2 (d).

Figure 6 depicts the frequency of alloreactive precursor helper T lymphocytes (pHLT) in fully MHC matched donor and stimulator cells incubated in a primary MLR in the presence of media alone, or with anti-B7-1 antibody (α-B7-1), an anti-B7-2 antibody (α-B7-2), anti-B7-1 and anti-B7-2 (αB7-1 + αB7-2), CTLA4Ig, or CIg.

Figure 7 depicts the frequency of alloreactive precursor helper T lymphocytes (pHLT) in MHC mismatched donor bone marrow treated with either irradiated allogeneic stimulator cells (host) or third party cells and CTLA4Ig or anti-ICAM.

Figure 8 depicts the frequency of alloreactive precursor helper T lymphocytes (pHLT) in MHC mismatched donor bone marrow treated with irradiated allogeneic stimulator cells and CTLA4Ig, control immunoglobulin (Ig) or cyclosporine A (CsA).

Figure 9 depicts the frequency of alloreactive precursor helper T lymphocytes (pHLT) in MHC mismatched donor bone marrow treated with irradiated allogeneic stimulator cells and anti-B7-1 antibody (αB7-1), anti-B7-2 antibody (αB7-2), anti-B7-1 and anti-B7-2 antibodies (α-B7-1 + α-B7-2), CTLA4Ig, or CIg.

Detailed Description of the Invention

This invention features methods for inhibiting antigen specific T cell responses *in vitro* by use of at least one agent which inhibits a costimulatory signal in T cells. As used herein, the phrase "inhibiting or inhibition of a T cell response" refers to a reduction in or substantial elimination of at least one T cell response, such as T cell proliferation, lymphokine secretion or induction of an effector function (e.g., induction of cytotoxic T cell activity or antibody production by B cells), upon exposure of the T cell to an antigen. The phrase "inhibiting or inhibition of a T cell response" is intended to encompass suppression of the response of a T cell to an antigen as well as induction of unresponsive in the T cell to the antigen, also referred to herein as induction of anergy in the T cell. A T cell which has been rendered unresponsive, or anergic, to a specific antigen exhibits substantially reduced or eliminated responses (e.g., proliferation and/or lymphokine production) upon reexposure to the antigen. In one embodiment of the invention, the response of a donor T cell to alloantigens is inhibited to reduce or substantially eliminate graft versus host disease in a bone marrow transplant recipient.

To inhibit a T cell response to an antigen according to the methods of the invention, a T cell is contacted with at least one inhibitor of a costimulatory signal in the T cell. An "inhibitor of a costimulatory signal" or an "agent which inhibits generation of a costimulatory signal" interferes with, blocks or substantially eliminates formation of or delivery of a second signal in the T cell which, together with a first, antigen specific, signal mediated through the TCR/CD3 complex, is necessary to induce an antigen specific response by the T cell. Typically, this second or costimulatory signal is mediated by a T cell surface receptor such as CD28 and/or CTLA4 (or related molecule) upon interaction with a ligand such as B7-1 and/or B7-2, (or related molecule, e.g., B7-3) on a cell presenting antigen to the T cell (e.g., on a B cell, on a "professional" antigen-presenting cell, or APC, such as a monocyte/macrophage, dendritic cell or Langerhans cell, or another cell type which can present antigen to a T cell, such as a keratinocyte, endothelial cell, astrocyte, fibroblast, or oligodendrocyte). Ligands such as B7-1 and B7-2 which trigger a costimulatory signal in a T

cell through a T cell surface receptor (e.g., CD28) are collectively referred to herein as "costimulatory molecules". T cell surface receptors to which such costimulatory molecules bind (e.g., CD28, CTLA4) are collectively referred to herein as "costimulatory receptors".

Accordingly, in one embodiment of the invention, an inhibitor of a costimulatory signal in a T cell is an agent which inhibits an interaction between a receptor on the T cell and a costimulatory molecule on a cell presenting antigen to the T cell. This type of agent, also referred to herein as a "costimulatory blocking agent" can be a soluble form of the receptor on the T cell (or a related receptor on the T cell which similar binding specificity), a soluble form of the costimulatory molecule(s), or an antibody (or fragment thereof) which binds to either the receptor or the costimulatory molecule. A preferred costimulatory inhibitor is CTLA4-immunoglobulin fusion protein (CTLA4Ig), a soluble form of the CTLA4 receptor on T cells which binds to both B7-1 and B7-2. An even more preferred costimulatory inhibitor is a combination of an anti-B7-1 and an anti-B7-2 antibody. In another embodiment, the costimulatory inhibitor acts intracellularly to inhibit generation of or delivery of a costimulatory signal in a T cell by a CD28- and/or CTLA4-associated signal transduction pathway.

These and other embodiments of the invention are described in further detail in the following subsections:

I. Agents for Inhibiting a T Cell Response

A. Antibodies

In one embodiment of the invention, an agent used to inhibit an antigen specific T cell response can be an antibody (or fragment thereof). Antibodies suitable for use in the methods of the invention are available in the art (e.g., from the American Type Culture Collection, Rockville, MD, or commercially, e.g., from Becton-Dickinson or Immunotech) or can be prepared by standard techniques for making antibodies. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen. Structurally, the simplest naturally occurring antibody (e.g., IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a naturally-occurring antibody. Thus, these antigen-binding fragments are also intended to be designated by the term "antibody". Examples of binding fragments encompassed within the term antibody include (i) an Fab fragment consisting of the VL, VH, CL and CH1 domains; (ii) an Fd fragment consisting of the VH and CH1 domains; (iii) an Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (iv) a dAb fragment (Ward et al., (1989) *Nature* 341:544-546) which consists of a VH domain; (v) an isolated complimentarity determining region (CDR);

and (vi) an $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described for whole antibodies. The term "antibody" is further intended to include bispecific and chimeric molecules having an antigen binding portion. Furthermore, although the two domains of an Fv fragment are coded for by separate genes, a synthetic linker can be made that enables them to be made as a single protein chain (known as single chain Fv (scFv); Bird et al. (1988) *Science* 242:423-426; and Huston et al. (1988) *PNAS* 85:5879-5883) by recombinant methods. Such single chain antibodies are also encompassed within the term "antibody".

To prepare an antibody specific for a molecule to be targeted in the method of the invention (e.g., a costimulatory molecule, an adhesion molecule, a growth factor receptor, etc.), an animal is immunized with an appropriate immunogen. The term "immunogen" is used herein to describe a composition typically containing a protein or peptide as an active ingredient used for the preparation of antibodies against the protein or peptide. It is to be understood that the protein or peptide can be used alone, or linked to a carrier as a conjugate, or as a peptide polymer. The immunogen should contain an effective, immunogenic amount of the peptide or protein (optionally as a conjugate linked to a carrier). The effective amount of the immunogen per unit dose depends on, among other things, the species of animal inoculated, the body weight of the animal and the chosen immunization regimen, as is well known in the art. The immunogen preparation will typically contain peptide concentrations of about 10 micrograms to about 500 milligrams per immunization dose, preferably about 50 micrograms to about 50 milligrams per dose. An immunization preparation can also include an adjuvant as part of the diluent. Adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and alum are materials well known in the art, and are available commercially from several sources.

Either soluble or membrane bound protein or peptide fragments are suitable for use as an immunogen. A purified form of protein, such as may be isolated from a natural source or expressed recombinantly by conventional techniques known in the art, can be directly used as an immunogen. Those skilled in the art will appreciate that, instead of using naturally occurring forms of protein for immunization, synthetic peptides can alternatively be employed towards which antibodies can be raised for use in this invention. The purified protein can also be covalently or noncovalently modified with non-proteinaceous materials such as lipids or carbohydrates to enhance immunogenicity or solubility. Alternatively, a purified protein can be coupled with or incorporated into a viral particle, a replicating virus, or other microorganism in order to enhance immunogenicity. It is also possible to immunize an animal with whole cells which express a protein on their surface against which an antibody is to be raised (e.g., T cells or antigen presenting cells expressing surface molecules of interest can be used as immunogens). As yet another alternative, it is possible to use nucleic acid (e.g., DNA) encoding the protein or peptide of interest as an immunogen for so-

called genetic immunization. Thus, the term "immunogen" is also intended to include nucleic acid encoding a protein or peptide against which antibodies are to be raised (see e.g., Tang, D.C. et al. (1992) *Nature* 356:152-154; Eisenbraun, M.D. et al. (1993) *DNA Cell Biol.* 12:791-797; Wang, B. et al. (1993) *DNA Cell Biol.* 12:799-805 for descriptions of genetic immunization).

Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of an immunogen and an adjuvant. As an illustrative embodiment, animals are typically immunized against a protein, peptide or derivative by combining about 1 μg to 1 mg of protein with Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of immunogen in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. Seven to 14 days later, the animals are bled and the serum is assayed for specific antibody titer (e.g., by ELISA). Animals are boosted until the titer plateaus. Also, aggregating agents such as alum can be used to enhance the immune response.

Such mammalian-produced populations of antibody molecules are referred to as "polyclonal" because the population comprises antibodies with differing immunospecificities and affinities for the immunogen. The antibody molecules are then collected from the mammal (e.g., from the blood) and isolated by well known techniques, such as protein A chromatography, to obtain the IgG fraction. To enhance the specificity of the antibody, the antibodies may be purified by immunoaffinity chromatography using solid phase-affixed immunogen. The antibody is contacted with the solid phase-affixed immunogen for a period of time sufficient for the immunogen to immunoreact with the antibody molecules to form a solid phase-affixed immunocomplex. The bound antibodies are separated from the complex by standard techniques.

The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site. A monoclonal antibody composition thus typically displays a single binding affinity for a particular protein with which it immunoreacts. Monoclonal antibodies can be prepared using a technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256:495-497; see also Brown et al. (1981) *J. Immunol* 127:539-46; Brown et al. (1980) *J Biol Chem* 255:4980-83; Yeh et al. (1976) *PNAS* 76:2927-31; and Yeh et al. (1982) *Int. J. Cancer* 29:269-75) and the more recent human B cell hybridoma technique (Kozbor et al. (1983) *Immunol Today* 4:72), EBV-hybridoma technique (Cole et al. (1985), Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96), and trioma techniques.

Thus, a monoclonal antibody can be produced by the following method, which comprises the steps of:

(a) Immunizing an animal with a protein (or peptide thereof). The immunization is typically accomplished by administering the immunogen to an immunologically competent mammal in an immunologically effective amount, i.e., an amount sufficient to produce an immune response. Preferably, the mammal is a rodent such as a rabbit, rat or mouse. The mammal is then maintained for a time period sufficient for the mammal to generate high affinity antibody molecules. Antibody production is detected by screening the serum from the mammal with a preparation of the immunogen protein. These screening methods are well known to those of skill in the art, e.g., enzyme-linked immunosorbent assay (ELISA) and/or flow cytometry.

(b) A suspension of antibody-producing cells removed from each immunized mammal secreting the desired antibody is then prepared. After a sufficient time, the animal (e.g., mouse) is sacrificed and somatic antibody-producing lymphocytes are obtained. Antibody-producing cells may be derived from the lymph nodes, spleens and peripheral blood of primed animals. Spleen cells are preferred, and can be mechanically separated into individual cells in a physiologically tolerable medium using methods well known in the art. Mouse lymphocytes give a higher percentage of stable fusions with the mouse myelomas described below. Rat, rabbit and frog somatic cells can also be used. The spleen cell chromosomes encoding desired immunoglobulins are immortalized by fusing the spleen cells with myeloma cells, generally in the presence of a fusing agent such as polyethylene glycol (PEG). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques; for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md.

The resulting cells, which include the desired hybridomas, are then grown in a selective medium, such as HAT medium, in which unfused parental myeloma or lymphocyte cells eventually die. Only the hybridoma cells survive and can be grown under limiting dilution conditions to obtain isolated clones. The supernatants of the hybridomas are screened for the presence of antibody of the desired specificity, e.g., by immunoassay techniques using the antigen that has been used for immunization. Positive clones can then be subcloned under limiting dilution conditions and the monoclonal antibody produced can be isolated. Various conventional methods exist for isolation and purification of the monoclonal antibodies so as to free them from other proteins and other contaminants. Commonly used methods for purifying monoclonal antibodies include ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography (see, e.g., Zola et al. in Monoclonal Hybridoma Antibodies: Techniques And Applications, Hurell (ed.) pp. 51-52 (CRC Press 1982)). Hybridomas produced according to these methods can be propagated *in vitro* or *in vivo* (in ascites fluid) using techniques known in the art.

Generally, the individual cell line may be propagated *in vitro*, for example in laboratory culture vessels, and the culture medium containing high concentrations of a single

specific monoclonal antibody can be harvested by decantation, filtration or centrifugation. Alternatively, the yield of monoclonal antibody can be enhanced by injecting a sample of the hybridoma into a histocompatible animal of the type used to provide the somatic and myeloma cells for the original fusion. Tumors secreting the specific monoclonal antibody produced by the fused cell hybrid develop in the injected animal. The body fluids of the animal, such as ascites fluid or serum, provide monoclonal antibodies in high concentrations. When human hybridomas or EBV-hybridomas are used, it is necessary to avoid rejection of the xenograft injected into animals such as mice. Immunodeficient or nude mice may be used or the hybridoma may be passaged first into irradiated nude mice as a solid subcutaneous tumor, cultured *in vitro* and then injected intraperitoneally into pristane primed, irradiated nude mice which develop ascites tumors secreting large amounts of specific human monoclonal antibodies.

Media and animals useful for the preparation of these compositions are both well known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM; Dulbecco et al. (1959) *Virol.* 8:396) supplemented with 4.5 gm/l glucose, 20 mM glutamine, and 20% fetal caf serum. An exemplary inbred mouse strain is the Balb/c.

When antibodies produced in non-human subjects are used therapeutically in humans, they are recognized to varying degrees as foreign and an immune response may be generated in the patient. One approach for minimizing or eliminating this problem, which is preferable to general immunosuppression, is to produce chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region. Such antibodies are the equivalents of the monoclonal and polyclonal antibodies described above, but may be less immunogenic when administered to humans, and therefore more likely to be tolerated by the patient.

Chimeric mouse-human monoclonal antibodies (i.e., chimeric antibodies) can be produced by recombinant DNA techniques known in the art. For example, a gene encoding the constant region of a murine (or other species) monoclonal antibody molecule is substituted with a gene encoding a human constant region. (see Robinson et al., International Patent Publication PCT/US86/02269; Akira, et al., European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al., PCT Application WO 86/01533; Cabilly et al. U.S. Patent No. 4,816,567; Cabilly et al., European Patent Application 125,023; Better et al. (1988 *Science* 240:1041-1043); Liu et al. (1987) *PNAS* 84:3439-3443; Liu et al. (1987) *J. Immunol.* 139:3521-3526; Sun et al. (1987) *PNAS* 84:214-218; Nishimura et al. (1987) *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al. (1988) *J. Natl Cancer Inst.* 80:1553-1559).

A chimeric antibody can be further "humanized" by replacing portions of the variable region not involved in antigen binding with equivalent portions from human variable regions.

General reviews of "humanized" chimeric antibodies are provided by Morrison, S. L. (1985) *Science* 229:1202-1207 and by Oi et al. (1986) *BioTechniques* 4:214. These methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of an immunoglobulin variable region from at least one of a heavy or light chain. Sources of such nucleic acid are well known to those skilled in the art and, for example, may be obtained from an anti-CTLA4 antibody producing hybridoma. The cDNA encoding the chimeric antibody, or fragment thereof, can then be cloned into an appropriate expression vector. Suitable "humanized" antibodies can be alternatively produced by CDR or CEA substitution (see U.S. Patent 5,225,539 to Winter; Jones et al. (1986) *Nature* 321:552-525; Verhoeyan et al. (1988) *Science* 239:1534; and Beidler et al. (1988) *J. Immunol.* 141:4053-4060).

As an alternative to humanizing an mAb from a mouse or other species, a human mAb directed against a human protein can be generated. Transgenic mice carrying human antibody repertoires have been created which can be immunized with human protein or peptide immunogen. Splenocytes from these immunized transgenic mice can then be used to create hybridomas that secrete human mAbs specifically reactive with the human protein (see, e.g., Wood et al. PCT publication WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. PCT publication WO 92/03918; Kay et al. PCT publication 92/03917; Lonberg, N. et al. (1994) *Nature* 368:856-859; Green, L.L. et al. (1994) *Nature Genet.* 7:13-21; Morrison, S.L. et al. (1994) *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman et al. (1993) *Year Immunol* 7:33-40; Tuaillon et al. (1993) *PNAS* 90:3720-3724; Bruggeman et al. (1991) *Eur J Immunol* 21:1323-1326; and).

Monoclonal antibodies can also be produced by other methods well known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal antibodies (for descriptions of combinatorial antibody display see e.g., Sastry et al. (1989) *PNAS* 86:5728; Huse et al. (1989) *Science* 246:1275; and Orlandi et al. (1989) *PNAS* 86:3833). After immunizing an animal with an immunogen as described above, the antibody repertoire of the resulting B-cell pool is cloned. Methods are generally known for directly obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of the heavy and light chain variable regions from a number of murine antibodies (Larrick et al. (1991) *Biotechniques* 11:152-156). A similar strategy can also been used to amplify human heavy and light chain variable regions from human antibodies (Larrick et al. (1991) *Methods: Companion to Methods in Enzymology* 2:106-110).

As an illustrative embodiment, RNA is isolated from activated B cells of, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. *PNAS* (1989) 86:3833-3837; Sastry et al., *PNAS* (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, the variable regions of both heavy and light chains are amplified, each alone or in combination, and ligated into appropriate vectors for further manipulation in generating the display packages. Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease recognition sequences may also be incorporated into the primers to allow for the cloning of the amplified fragment into a vector in a predetermined reading frame for expression.

The V-gene library cloned from the immunization-derived antibody repertoire can be expressed by a population of display packages, preferably derived from filamentous phage, to form an antibody display library. Ideally, the display package comprises a system that allows the sampling of very large diverse antibody display libraries, rapid sorting after each affinity separation round, and easy isolation of the antibody gene from purified display packages. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating a variegated antibody display library can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. International Publication No. WO 92/18619; Dower et al. International Publication No. WO 91/17271; Winter et al. International Publication WO 92/20791; Markland et al. International Publication No. WO 92/15679; Breitling et al. International Publication WO 93/01288; McCafferty et al. International Publication No. WO 92/01047; Garrard et al. International Publication No. WO 92/09690; Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J Mol Biol* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

Once displayed on the surface of a display package (e.g., filamentous phage), the antibody library is screened with a protein, or peptide fragment thereof, to identify and isolate packages that express an antibody having specificity for the protein. Nucleic acid encoding the selected antibody can be recovered from the display package (e.g., from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques.

In another embodiment of the phage display library screening approach, the V region domains of heavy and light chains are expressed on the same polypeptide, joined by a flexible linker to form a single-chain Fv fragment, and the scFV gene is subsequently cloned into the desired expression vector or phage genome. As generally described in McCafferty et al., *Nature* (1990) 348:552-554, complete V_H and V_L domains of an antibody, joined by a flexible $(Gly_4\text{-}Ser)_3$ linker can be used to produce a single chain antibody which can render the display package separable based on antigen affinity. Isolated scFV antibodies immunoreactive with a particular antigen can subsequently be formulated into a pharmaceutical preparation for use in the subject method.

B. Soluble Proteins and Fusion Proteins

In another embodiment of the invention, an agent used to inhibit a T cell response is a soluble form of a costimulatory receptor on the surface of a T cell, or a a costimulatory molecule on the surface of a cell which presents antigen to the T cell. This soluble protein is capable of inhibiting an interaction between the surface form of the molecule and its ligand(s) (and/or inhibiting an interaction between a related surface molecule having similar binding specificity and its ligand (s)). For example, soluble forms of CTLA4, B7-1 and or B7-2 can be used. A preferred agent for use in the inhibiting donor T cell responses in bone marrow transplantation is a soluble form of a CTLA4 molecule (in particular, a CTLA4-immunoglobulin fusion protein) which binds to both B7-1 and B7-2, and can inhibit the interaction of B7-1 and B7-2 with CD28 and/or CTLA4.

Soluble forms of surface-bound proteins can be made using standard recombinant DNA and protein expression techniques known in the art. Nucleic acid comprising a nucleotide sequence encoding the extracellular domain (or portion thereof) of a surface-bound protein of interest (i.e., lacking the nucleotide sequence of the transmembrane and cytoplasmic domains) can be isolated and cloned into a standard expression vector, either for expression in prokaryotic or eukaryotic cells. The expression vector is introduced into an appropriate host cell (e.g., *E. coli* for prokaryotic expression; yeast or mammalian cells, e.g., COS, CHO or NS0 cells, for eukaryotic expression) and the cells are cultured to allow for expression of the protein encoded therein. The protein is then purified by standard techniques from harvested host cells or, if the protein is secreted from the cells, from the media in which the cells are cultured.

The extracellular domain (or portion thereof) of a surface-bound protein can be expressed recombinantly as a non-fusion protein, or more preferably, is expressed as a fusion protein with a second protein or polypeptide. As used herein, the term "fusion protein" refers to a protein composed of a first polypeptide operatively linked to a second, heterologous, polypeptide. A preferred type of fusion protein to be used as an agent in the methods of the invention is an immunoglobulin fusion protein (e.g., CTLA4Ig). The term "immunoglobulin fusion protein" refers to a fusion protein in which the second, heterologous polypeptide is an

immunglobulin constant region, or portion thereof. Immunoglobulin fusion proteins have been described extensively in the art (see e.g., U.S. Patent No. 5,116,964 by Capon et al.; Capon, D.J. et al. (1989) *Nature* 337:525-531; and Aruffo, A. et al. (1990) *Cell* 61:1303-1313), and typically include at least a functionally active hinge region, CH_2 and CH_3 domains of a constant region of an immunoglobulin heavy chain (e.g., human $C\gamma1$, $C\gamma4$). Construction of a B7-1-Ig fusion protein and a CD28Ig fusion protein is described in detail in Linsley, P.S. et al. (1991) *J. Exp. Med.* 173:721-730. Construction of a CTLA4Ig fusion protein is described in detail in Linsley, P.S. et al. (1991) *J. Exp. Med.* 174:561-569 and Gimmi, C.D. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:6586. Other Ig fusion proteins (e.g., B7-2-Ig) can be similarly prepared.

C. Additional Blocking Agents

Alternative to an antibody (or fragment thereof), soluble receptor or ligand (or portion thereof), other molecules which inhibit interactions between cell surface molecules are within the scope of the invention for use in inhibiting T cell responses. For example, a peptide, peptide mimetic, or other form of small molecule (such as a drug) which inhibits an interaction between a receptor and a costimulatory molecule can be used to inhibit a costimulatory signal in a T cell. Similarly, a peptide, peptide mimetic, or other form of small molecule (such as a drug) which inhibits adhesion or a T cell to a cell presenting antigen to the T cell, or inhibits an interaction between a T cell growth factor and its receptor on a T cell, can be used as a second agent in conjunction with a costimulation inhibitory agent to inhibit a T cell response.

D. Intracellular Agents

In other embodiments of the described methods, an agent which acts intracellularly to interfere with the formation of an intracellular signal(s) associated with a particular signal transduction pathway can be used to inhibit a T cell response. For example, a costimulation inhibitory agent as described herein can be an agent that acts intracellularly to inhibit a CD28- or CTLA4-associated signal transduction pathway. CD28 stimulation has been shown to result in protein tyrosine phosphorylation in T cells (see e.g., Vandenberghe, P. et al. (1992) *J. Exp. Med.* 175:951-960; Lu, Y. et al. (1992) *J. Immunol.* 149:24-29). Accordingly, a tyrosine kinase inhibitor, such as herbimycin A, can be used to inhibit a CD28-associated signal transduction pathway, thereby inhibiting generation of a costimulatory signal in the T cell. Alternatively, a CD28-associated signal transduction pathway can be inhibited using an agent which stimulates protein tyrosine phosphatase activity in a T cell, thereby decreasing the net amount of protein tyrosine phosphorylation. For example, an antibody directed against the cellular tyrosine phosphatase CD45 can be used to stimulate tyrosine phosphatase activity in a T cell expressing CD45 on its surface. Other intracellular signals reported to be associated with CD28 ligation include increased phospholipase C activity (see e.g., Nunes, J.

et al. (1993) *Biochem. J.* 293:835-842) and increased intracellular calcium levels (see e.g. Ledbetter, J.A. et al. (1990) *Blood* 75:1531-1539). Accordingly, an agent which inhibits phospholipase C activity and/or inhibits increases in intracellular calcium levels can be used to inhibit the generation of a costimulatory signal in a T cell.

E. Compositions

The agents used *in vitro* according to the described methods to inhibit a T cell response can be formulated into pharmaceutical compositions suitable for administration *in vivo* to a bone marrow transplant recipient following transplantation. Accordingly, another aspect of the invention pertains to pharmaceutical compositions. A preferred composition of the invention comprises a CTLA4Ig fusion protein, in an amount effective to inhibit a T cell response, and a pharmaceutically acceptable carrier.

The agents of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo* to inhibit a T cell response. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the ligand. The term subject is intended to include living organisms in which an immune response can be elicited, e.g., mammals. Examples of subjects include humans, monkeys, dogs, cats, mice, rats, and transgenic species thereof.

Administration of a therapeutically active amount of one or more of the agents described herein is defined as an amount effective, at dosages and for periods of time necessay to achieve the desired result. For example, a therapeutically active amount of a CTLA4Ig fusion protein may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the fusion protein to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active agent (e.g., antibody and/or fusion protein) may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. To administer an agent by other than parenteral administration, it may be necessary to coat the agent with, or co-administer the agent with, a material to prevent its inactivation. An agent may be administered to an individual in an appropriate carrier or diluent, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional

liposomes (Strejan et al., (1984) *J. Neuroimmunol* 7:27). Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, an isotonic buffered saline solution, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active agent in the required amount of an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (e.g., protein) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the active compound is suitably protected, as described above, the compound may be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

II. Uses of the Invention

The methods of the invention can be used to inhibit T cell responses *in vitro* by contacting a T cell with a costimulation inhibitory agent as described herein. Accordingly, the term "contacting" as used herein is intended to include incubating (or culturing) a T cell with the agent. The methods of the invention are useful in therapeutic situations where it is desirable to inhibit an unwanted T cell response, as described in further detail in the subsections to follow. Additionally, in one embodiment, the methods of the invention induce antigenic nonresponsiveness in a T cell that persists after cessation of treatment (i.e., antigenic nonresponsiveness persists *in vivo* after contacting T cells *in vitro* with a costimulation inhibitory agent). Thus, the methods of the invention are useful for inducing T cell anergy, thereby providing a means for long-term inhibition of T cell responses without the need for chronic generalized immunosuppression of a subject with its attendant deleterious side effects.

The methods of the invention are particularly useful for inhibiting graft versus host disease which results from allogeneic bone marrow transplantation. It has previously been observed that the presence of mature donor T cells within a bone marrow graft is beneficial both for successful engraftment and for a graft versus leukemia response. However, the presence of mature donor T cells in the graft induces GVHD. Responses of alloreactive donor T cells can be inhibited by contacting the T cells *in vitro* with a costimulation inhibitory agent (e.g., a costimulation blocking agent, such as a combination of an anti-B7-1 and an anti-B7-2 antibody, or CTLA4Ig). Such treatment allows mature T cells to be present within transplanted donor cells, thus avoiding GVHD and promoting bone marrow engraftment. Moreover, T cell unresponsiveness to alloantigens is induced, thereby providing long-term inhibition of T cell responses without the need for continuous treatment of the bone marrow recipient.

Because the T cells to be inhibited in a bone marrow transplant situation are donor T cells which are available *in vitro* prior to transplantation, alloreactive donor T cell responses can be be inhibited *in vitro*, or inhibited *in vitro* followed by an *in vivo* treatment regiment. Accordingly, graft versus host disease in a bone marrow transplant recipient is inhibited by

contacting a population of donor T cells *in vitro* (prior to transplantation) with 1) a second population of cells expressing recipient alloantigens (such as recipient cells or cells from another source which share recipient alloantigens, e.g., major or minor histocompatibility antigens) and 2) an agent which inhibits a costimulatory signal in a donor T cell. In one embodiment, the agent which inhibits a costimulatory signal is a CTLA4Ig fusion protein. In another embodiment, the agent is an anti-B7-1 or anti-B7-2 antibody (or fragment therof). In a much preferred embodiment, the agent is a combination of an anti-B7-1 antibody and an anti-B7-2 antibody. In yet another embodiment the agent is a single antibody which binds both B7-1 and B7-2. The second population of cells, which express recipient alloantigens, are typically treated such that they cannot proliferate and/or are not metabolically active, e.g., the cells are irradiated and/or treated with paraformaldehyde.

In the method, the population of donor cells contacted with the inhibitory agent(s) include mature donor T cells. Accordingly, the population of donor cells used in the method can be, for example, the bone marrow cells themselves which are to be transplanted into the recipient which have not been T cell depleted. Alternatively, or additionally, the source of mature donor T cells can be donor peripheral blood cells, splenocytes or other suitable source of donor T cells. When non-bone marrow T cells (e.g., peripheral blood T cells or splenocytes) are used as the source of mature T cells which are contacted with the inhibitory agent(s), the subsequent bone marrow graft includes a mixture of bone marrow cells and non-bone marrow cells (i.e., bone marrow cells together with mature donor T cells in which alloreactivity has been inhibited). Following *in vitro* culture of donor cells with cells expressing recipient alloantigens and inhibitory agent(s), the donor cells are administered to the recipient (if the donor cells used in the *in vitro* culture do not include bone marrow cells, e.g., if peripheral blood cells or splenocytes are uses as the source of mature donor T cells, then T-cell depleted bone marrow cells are also administered to the recipient).

In another embodiment, following *in vitro* treatment and administration of donor cells to the recipient, the recipient is further treated *in vivo* with the inhibitory agent(s). That is, a costimulation inhibitory agent can be administered to the recipient alone or with a another agent, such as an immunosupressive agent (e.g., cyclosporine A) or only the immunosupressive agent can be administered *in vivo*.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLE 1: Inhibition of Responses of Alloreactive T Cells by CTLA4Ig

(A) This experiment demonstrated that the addition of CTLA4Ig to a human fully disparate, primary mixed lymphocyte reaction (MLR) has been shown to completely inhibit the proliferation of the responder T cells and to abrogate the production of IL-2. In this

experiment, 1 x 10^5 responder T cells were mixed with 1 x 10^5 irradiated MHC disparate PBLs in the absence or presence of CTLA4Ig or an isotype matched control Ig protein each present at 10 μg/ml. An additional MLR was set up containing cells only and lacking any CTLA4Ig or control Ig protein. Portions of the culture were removed daily from day 1 through 8 and pulsed overnight with [^{3}H]-thymidine to measure proliferation of the responder T cells. As is shown in Figure 1, treatment of the MLR with control Ig protein had no effect on the alloantigen driven proliferation of the responder T cells. Both the control Ig and untreated cultures exhibit strong proliferation as is epected between cells from MHC disparate individuals and show a peak proliferative response on around day six. In contrast, the addition of CTLA4Ig abrogates most of the proliferative response to the stimulator cells and proliferation never rises above a basal level. Both the untreated and the control Ig treated cultures produced large amounts of IL-2 (~ 1000 pg/ml), a cytokine known to be required for the T cell proliferation. The CTLA4Ig treated cultures failed to produce any detectable IL-2 (<20 pg/ml).

In another experiment shown in Figure 2, primary MLR between HLA-disparate individuals are inhibited only some 30% by the addition of a monoclonal antibody that block either B7-1, whereas anti-B7-2 inhibited maximal proliferation by 70%. The combination of anti B7-1 and anti-B7-2 mAbs (10 μg/ml) reduced proliferation by greater than 90% and also completely abrogated IL-2 accumulation in the supernatant. The addition of CTLA4-Ig results in greatly increased inhibition of proliferation in primary MLR (approximatetly 90%). In contrast, control-Ig has no effect. The combination of anti-B7-1 and anti-B7-2 was more efficient at blocking peak proliferation than wither CTLA4-Ig ($p<0.05$) or anti-B7-2 mAb alone ($p<0.005$).

(B) This experiment demonstrates the utility of CTLA4Ig in a bone marrow derived system. In this system, donor bone marrow (5 x 10^4 cells) was mixed with NIH3T3 cells previously transfected with either human B7-1 or human B7-2 (5 x 10^4) in the presence of submitogenic amounts of an immobilized anti-CD3 monoclonal antibody or the donor bone marrow (1 x 10^5 cells) was mixed with PBLs (1 x 10^5) obtained from a fully MHC disparate individual. The stimulator cells had been previously inactivated by treatment with mitomycin C or by irradiation before addition. All of these experiments were performed in the absence or presence of graded concentrations of CTLA4Ig for the entire incubation period. In assays where the costimulation was provided by the transfected cells, the cultures were pulsed with [^{3}H]-thymidine overnight between days 2 and 3 of culture. In assays where the costimulation was provided by the allogeneic PBLs, the cultures were pulsed with [^{3}H]-thymidine overnight between days 4 and 5 of culture. The amount of radioactivity incorporated into the dividing responder T cells determined their level of proliferation. These time points were chosen because they had been previously shown to be at or near the proliferation maxima. The results of these experiments are shown in the Figures 3A and 3B.

where the line labeled PBLs represents the data obtained with the allogeneic stimulators and the line labeled t/B7-1 or t/B7-2 represent the two transfected cell lines as costimulators. This data represents the mean data from six experiments.

Figure 3A shows the inhibition of donor marrow proliferation by amounts of CTLA4Ig below 1μg/ml. Treatment with 0.01 μg/ml of CTLA4Ig had no effect on the proliferative response of the donor bone marrow. Treatment at doses from 0.1 to 1.0 μg/ml greatly reduced the proliferative response to the two transfected cell lines and reduced the proliferative response to the allogeneic PBLs to background levels. Figure 3B shows inhibition of T cell proliferative responses at CTLA4Ig treatment levels from 1 to 10 μg/ml. The proliferative response to the human B7-1 transfected cells is completely inhibited by CTLA4Ig levels of between 2 and 5 μg/ml. The proliferative response to the B7-2 transfected cells and to the allogeneic PBLs is completely inhibited at CTLA4Ig levels of 2 μg/ml and 1 μg/ml, respectively. Since the proliferation driven by the allogeneic PBL was almost completely inhibited by 1 μg/ml of CTLA4Ig and was completely inhibited by 2 μg/ml CTLA4Ig, a dosage of 10 μg/ml of CTLA4Ig was selected for use in all subsequent experiments.

EXAMPLE 2: Blockade of B7 family mediated costimulation specifically inhibits proliferation upon rechallenge with donor but not third party alloantigen

To determine whether agents which block proliferation in a primary MLR inhibit proliferation upon rechallenge in a secondary MLR, the following experiment was performed.

Normal peripheral blood cells mononuclear cells were cultured with irradiated (2500cGy) normal donor peripheral blood mononuclear cells of fully HLA mismatched subject in the presence of an anti-MHC class II antibody, an anti-ICAM-1 antibody, cyclosporin A (CsA), CTLA4Ig, an anti-B7-1 antibody, an anti-B7-2 antibody, or a combination of an anti-B7-1 and an anti-B7-2 antibody at a final concentration of 10^6 cells/ml. The antibodies and CTLA4Ig were added at a concentration of 10 μg/ml. Cyclosporin A was used at 10^{-3} M. The cells were incubated for 6 days, following which viable cells were isolated and rechallenged with irradiated allogeneic cells from the original donor or from a third party donor. No blocking agents were added to this secondary MLR. Proliferation was examined daily for 7 days by $[^3H]$-thymidine incorporation assays.

The peak proliferation for each condition are represented in Figure 4. The results indicate that when only media had been added to the primary MLR, a typical secondary response ensued characterized by significantly greater and earlier peak proliferation against donor compared with third party alloantigen (peak at day 3 versus day 6, respectively). Addition of CsA to the primary MLR prevented the secondary response and resulted in

decreased levels of peak proliferation against both donor and third party on day 5. Addition of anti-Class II or anti-ICAM-1 mAbs during the primary MLR resulted in equivalent levels of secondary proliferation against both donor and third party that peaked on day 5 and 6. The absence of a secondary response following blockade of antigen recognition or adhesion demonstrates that alloreactive T cells to alloantigen. In contrast, complete blockade of B7 family mediated costimulation during the primary MLR with either CTLA4-Ig or the combination of anti B7-1 and anti-B7-2 resulted in markedly decreased proliferation to donor but had no effect against third party. Therefore, complete blockade of B7 family mediated costimulation but with intact alloantigen recognition and adhesion is necessary to induce alloantigen specific anergy.

EXAMPLE 3: **Maximal hyporesponsiveness induced by blockade of B7-1 and B7-2 costimulation is achieved within 36 hours**

This example analyses the time of inhibition of costimulation required to obtain maximal hyporesponsiveness for different agents blocking costimulation.

In this example, the same assay as that used in Example 2 was used. Cells were cultured in a primary MLR with the addition of anti-B7-1, anti-B7-2, a combination of anti-B7-1 and anti-B7-2, or CTLA4Ig. After various times, viable cells were isolated, re-challenged with irradiated cells from the original donor with no blocking agent added and proliferation assessed daily for 7 days. Peak proliferation obtained on any day in the secondary MLR was assessed by [^{3}H]-thymidine incorporation.

The results are presented in Figure 5. The results indicate that addition of anti-B7-1 mAb in the primary MLR resulted in only modest inhibition of proliferation on rechallenge whereas the addition of anti-B7-2 mAb was significantly more efficient. The maximal effect of anti-B7-2 mAb occurred by 36 hour in primary culture whereas with anti-B7-1 mAb this was not observed until day 6. Maximal hyporesponsiveness required blockade of both B7-1 and B7-2 by the addition of either anti-B7-1 and anti-B7-2 mAbs or CTLA4-Ig and was achieved within 36 hours. Addition of isotype matched control mAb or control-Ig fusion protein had no effect.

Thus, maximal hyporesponsiveness was obtained by incubating the cells for 36 hours with anti-B7-1 and anti-B7-2 antibodies.

EXAMPLE 4: **Reduction In The Frequency Of Alloreactive Precursor Helper T Cells With CTLA4Ig**

This experiment assays whether CTLA4Ig or anti-B7-1 and/or anti-B7-2 antibodies used to induce anergy in donor T cells to the alloantigens on recipient stimulator cells would

lead to the development of a subsequent hypo responsiveness to the stimulator cells without affecting the ability of the donor T cells to respond to third party antigens.

Non-reactivity in a matched sibling MLR is a poor predictor of subsequent GVHD. In contrast, in HLA identical sibling BMT recipients, a high frequency of alloreactive donor pHTL is associated with subsequent development of acute GVHD (Theobald, M., et al. (1992) *N. Engl. J. Med.* 327, 1613; Schwarer, A.P, et al. (1993) *Lancet* 341, 203). Therefore, the frequency of precursor Helper T Lymphocytes (pHTL) in HLA matched siblings following blockade of B7 family mediated costimulation during the primary culture was analyzed.

Stimulator cells at 10^6 cells/well were irradiated (25 Gy) and mixed with 10^5 unirradiated responder cells from fully HLA matched siblings. After incubation of the cells for 36 hours in the presence of media alone or with anti-B7-1 mAb, anti-B7-2 mAb, anti-B7-1 mAb and anti-B7-1 mAb, CTLA4Ig, or control immunoglobulin (CIg), the cells were irradiated (25Gy) and CTLL-2 cells were added and the culture was continued for a further 24 hours. Proliferation was assessed by tritiated thymidine incorporation for the last 16 hours or the assay. pHTL frequencies were calculated from the proportion of wells negative for IL-2 production by limiting dilution analysis (Taswell C. (1981) *J. Immunol.* 126, 1614).

The results are presented in Figure 6. As reported by others, prior to any manipulation the pHTL frequency in fully HLA matched pairs ranged from 1.25×10^{-4} to 8×10^{-7}. Culture with media during primary stimulation did no alter this frequency. Complete blockade of B7 family mediated costimulation by either the addition of the combination of anti-B7-1 and anti-B7-2 or CTLA4-Ig during primary stimulation markedly reduced the pHTL frequency against donor on rechallenge. In contrast, the addition of anti-B7-1 mAb or control Ig had no effect whereas anti-B7-2 had very modest effect. The pHTL frequencies achieved with complete B7 blockade were consistently $<1 \times 10^5$ that is below the level associated with development of GVHD.

Since the presence of anti-B7-1 and anti-B7-2 antibodies or CTLA4Ig in MLRs from fully HLA matched siblings results in reduction of the pHTL frequency against donor on rechallenge, the effect of these agents on pHTL frequency was assessed in MLR from fully HLA mismatched individuals. Approximately 40 different pairs of MHC mismatched combinations of donor bone marrow and irradiated allogeneic stimulator cells were tested as follows. Donor marrow and stimulator cells were incubated for 48 hours in the presence of either CTLA4Ig, isotype matched control Ig protein, anti-ICAM monoclonal antibody, or cyclosporine A. The results of these experiments are shown in the Figure 7 and 8. Incubation of the donor bone marrow, stimulator cell pairs in the presence of media, control Ig cyclosporine A, or CTLA4Ig for either 36 or 48 hour periods had no effect on the survival of bone marrow cells. Identical numbers of cells and cell types were present after treatment as in the untreated, freshly prepared marrow. However, following treatment of the donor marrow/stimulator cell mixture with CTLA4Ig, the frequency of alloreactive precursor helper

BFU-E

BFU-E/100,000 bone marrow cells

TABLE 1

Direct assay							
Patient #	Pre treatment	Media		Control Ig		CTLA4Ig	
		Mean	SD	Mean	SD	Mean	SD
1	232	ND	ND	425.3	109.3	453.3	148.9
2	293	302	37.3	340.7		291.3	29.3
3	570	578.7	63.3	510.7	38.4	608.7	20.8
4	456	389.3	18.8	376.7	45.8	429.3	60.1
5	416	495.3	59.9	512.7	30.8	515.3	23.1
6	593	597.3	38.6	408	30.3	610	44.3

LTCIC assay							
Patient #	Pre treatment	Media		Control Ig		CTLA4Ig	
		Mean	SD	Mean	SD	Mean	SD
1	89	77.8	11.4	94	6	84.8	2.3
2	238	170	23	132.7	20.7	206	23.2
3	560	548	56	500	34.7	567	13.7

CFU-GM

CFU-GM/100,000 bone marrow cells

Direct assay							
Patient #	Pre treatment	Media		Control Ig		CTLA4Ig	
		Mean	SD	Mean	SD	Mean	SD
1	122	ND	ND	121.3	6.4	139.3	20.3
2	167	174	5.3	147.3	38.7	16.7	22.2
3	84	68.7	7.4	96	1	98.7	18
4	108	154	21.3	130.7	39	120.7	17.6
5	74	86.7	26.9	63.3	12	45.3	8.8
6	36	36.7	10.4	34.7	11.1	39.3	4

LTCIC assay							
Patient #	Pre treatment	Media		Control Ig		CTLA4Ig	
		Mean	SD	Mean	SD	Mean	SD
1	12	11.2	4.8	114.3	2	14.2	1
2	58	110.7	6	66	11	81.3	8.3
3	114	156	22.7	128	9.3	130.7	10.2

T cells (pHTL) is reduced. This reduction in the frequency of the pHTL in the donor marrow anergized by treatment with CTLA4Ig is important because it has been demonstrated that the development of GVHD after bone marrow transplantation is directly associated with the frequency of the population of T cells. Donor pHTL frequencies were calculated using a limiting dilution assay (LDA) in which dilutions of donor cells were incubated with stimulator or third party cells in microtiter dishes. The supernatants from these cultures were then tested for their ability to support the proliferation of an IL-2 dependent CTLL cell line. This assay is very sensitive and measures the production of IL-2 by the treated donor marrow cells in response to various stimulators. The proliferation of the CTLL cell line is dependent on IL-2.

Figure 7 shows the pHTL frequency found in the donor marrow after anergization for 48 hours in the presence of 10 μg /ml of CTLA4Ig. Each point in this figure represents the data obtained from a different donor marrow MHC mismatched allogeneic stimulator cell pair. Untreated cells incubated in media alone show a pHTL frequency of approximately 10^{-2} when tested against the original stimulator cell population. This agrees with the pHTL frequency expected for a mismatched responder and stimulator pair. In contrast, donor marrow anergized by treatment with CTLA4Ig shows a dramatic reduction in the pHTL frequency when tested against the original stimulator cells (approximately 10^{-5}). The reduction in pHTL frequency is approximately 1000 fold. The specificity of the reduction in pHTL frequency towards the allogeneic stimulator cells is shown by measuring the pHTL frequency against third party PBLs which are MHC disparate with both the bone marrow and the original stimulator cells. The frequency of pHTL directed against the third party cells is approximately 10^{-2}. The pHTL frequency determined for the response of anergized donor marrow against third party cells is comparable with that found for untreated marrow. This demonstrates that the anergization of the donor marrow by exposure to allogeneic stimulators in the presence of CTLA4Ig results in the generation of a hypo responsiveness to the tolerizing stimulator cells but has no effect on the responsiveness to other alloantigens. The specificity of this induction of alloantigen hypo responsiveness by the inhibition of costimulation with CTLA4Ig was shown by testing the effect of a monoclonal antibody to the cell adhesion molecule ICAM on the pHTL frequency. Incubation of the donor marrow and stimulator cells cultures in the presence of 10 μg/ml of anti-ICAM monoclonal antibody had no effect on the pHTL frequency either to the original stimulator cells or to third party cells. The pHTL frequency to both cell types is comparable to that found for untreated marrow (10^{-2}).

As shown in Figure 8, the addition of isotype matched control Ig protein (10 μg/ml) or cyclysporine A to the donor marrow, stimulator cell cultures during the entire incubation period had no effect on the pHTL frequency when tested against the original stimulator cells. The pHTL frequency (10^{-2}) was comparable to that for untreated cultures demonstrating that the dramatic reduction seen in pHTL frequency in cultures treated with CTLA4Ig was not

due to the effect of the Ig sequences, nor was it due to a general immuosuppression as can be obtained with cyclosporine A.

In another example, the efficiency of anti-B7-1, anti-B7-2, or of a combination of anti-B7-1 and anti-B7-2 in decreasing the pHTL frequency against fully HLA mismatched donor alloantigen on rechallenge was examined. This experiment was performed as indicated above. The results are presented in Figure 9. The addition of either anti-B7-1 or anti-B7-2 mAbs alone had only modest effect on pHTL frequency. However, their combination was highly effective and consistently greater than blockade with CTLA4-Ig. These results demonstrate consistent reduction of the pHTL frequency for fully mismatched donors to levels below that associated with development of GVHD when using a combination of anti-B7-1 and anti-B7-2 antibodies. Of interest, although addition of CsA in the primary culture inhibited proliferation during secondary challenge to both donor and third party alloantigen, no effect on pHTL frequency was observed. When both CsA and CTLA4-Ig were added to the primary culture, no decrease in pHTL frequency was seen in distinct contrast tot the result observed with CTLA4-Ig alone. These observations are consistent with the hypothesis that there is a requirement for both a TCR mediated signal and blockade of B7 family mediated costimulation to induce alloantigen specific anergy.

In conclusion, treatment of donor marrow, stimulator cell cultures with a combination of anti-B7-1 and anti-B7-2 antibodies or with CTLA4Ig was shown to have no effect on the total number of cells recovered after the incubation protocol and to induce a dramatic alloantigen specific hypo responsiveness to the stimulator cells. Third party responsiveness remains at normal levels in the anti-B7-1 and anti-B7-2 or CTLA4Ig treated marrow. These studies show that the anergization of donor marrow with a combination of anti-B7-1 and anti-B7-2 antibodies or with CTLA4Ig will not have any effect on the donor cells except to anergize those cells to the stimulator alloantigens. The level of reduction in pHTL frequency after treatment with anti-B7-1 and anti-B7-2 antibodies or CTLA4Ig is consistent with a reduction in pHTL sufficient to reduce or eliminate GVHD. Furthermore, the combination of anti-B7-1 and anti-B7-2 antibodies was consistently more efficient than CTLA4 in blocking proliferation in a primary and a secondary MLR with fully HLA mismatched donor and recipient cells and in decreasing pHTL frequencies.

EXAMPLE 5: CTLA4Ig Has No Effect On Cell Lineage Precursor Or Stem Cells

This experiment was conducted to determine the effect of treatment with CTLA4Ig on cell lineage precursor or stem cells present in the donor marrow population. Colony forming assays and long term colony initiating cell (LTCIC) assays were performed on donor marrow before treatment, after incubation in media alone or in the presence of control Ig protein (10 μg/ml) or CTLA4Ig (10 μg/ml). Briefly, the marrow sample was washed extensively, the viable cell number determined, and the cells plated at 100,000 cells/ml in methylcellulose

medium which contained growth factors appropriate for human cells. These assays were set up in triplicate for each patient's marrow sample. After 10 days of incubation, the number of erythrocytic (BFU-E) and myelomonocytic (CFU-GM) colonies were counted. The LTCIC colony forming assays were performed using a limiting dilution analysis. The donor bone marrow sample was washed and dilutions from 40,000 to 625 cells were added to microtiter wells previously seeded with 3000 irradiated stromal cells. After 5 weeks in culture, the cells were gently trypsinized to release cells and then methylcellulose medium containing growth factors was added to each well. The appearance and number of BTU-E and CFU-GM colonies was measured after an additional 10 days of incubation. These results are shown in Table I for six of the patient samples.

The direct colony forming assay shows that there are similar numbers of BFU-E and CFU-GM precursors in all six patient samples tested regardless of whether the assays were performed on donor marrow before treatment or after culturing donor marrow with irradiated stimulators for 48 hours in the presence of medium alone or in medium containing control Ig or CTLA4Ig. Similar results are seen in the three patient samples tested by the LTCIC methodology. This method is a more sensitive measurement of damage to stem cells than is the direct colony forming assay. All three patient samples tested have similar numbers of BFU-E and CFU-GM colony forming units after treatment as did the fresh donor marrow samples.

The experimental data presented in Table I shows that the culturing protocol developed for the anergization of donor bone marrow to sensitizing alloantigens by the treatment with CTLA4Ig had no detrimental effect on the donor bone marrow. The donor bone marrow had the same number of cells before and after treatment. The donor bone marrow retained a full complement of lineage specific blood cell precursors as demonstrated in both direct colony forming assays and in the more sensitive LTCIC assays. However, the donor bone marrow exhibited a dramatic and specific decrease in the responsiveness to the sensitizing alloantigens while it retained a full ability to respond to third party cells. This loss of responsiveness of the donor bone marrow to the sensitizing cells after treatment with CTLA4Ig was as expected based on previous *in vitro* and *in vivo* studies. The loss of donor marrow responsiveness to the sensitizers suggests that transplantation of similarly treated marrow into the appropriate recipient will be safe for the transplant recipient and may result in a reduction or elimination of acute GVHD in the transplant recipient.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for inhibiting a response by a T cell to an antigen, comprising contacting the T cell with and agent which inhibits a costimulatory signal in the T cell, thereby inhibiting the response by the T cell to the antigen.

2. The method of claim 1, wherein the agent inhibits an interaction between a receptor on the T cell and a costimulatory molecule on a cell presenting antigen to the T cell.

3. The method of claim 2, wherein the receptor on the T cell is CD28.

4. The method of claim 2, wherein the receptor on the T cell is CTLA4.

5. The method of claim 2, wherein the costimulatory molecule is B7-1 or B7-2.

6. The method of claim 2, wherein the agent is a soluble form of CTLA4.

7. The method of claim 6, wherein the soluble form of CTLA4 is a human CTLA4-immunoglobulin fusion protein.

8. The method of claim 2, wherein the agent is an anti-B7-1 antibody, or fragment thereof.

9. The method of claim 2, wherein the agent is an anti-B7-2 antibody, or fragment thereof.

10. The method of claim 2, wherein the agent is an anti-B7-1 antibody and an anti-B7-2 antibody, or fragments thereof.

11. The method of claim 1, wherein the agent acts intracellularly to inhibit generation of a costimulatory signal in the T cell.

12. A method for inhibiting graft versus host disease in a bone marrow transplant recipient, comprising contacting a first population of cells comprising donor T cells *in vitro* with:

a) a second population of cells which express recipient alloantigens; and

b) an agent which inhibits a costimulatory signal in the donor T cells, the agent thereby inhibiting a response by the donor T cells to the cells which express recipient alloantigens such that, upon administration of the first population of cells to the bone marrow transplant recipient, graft versus host disease in the recipient is inhibited.

13. The method of claim 12, wherein the first population of cells is selected from a group consisting of bone marrow cells, peripheral blood cells and splenocytes.

14. The method of claim 12, wherein the agent is a soluble form of CTLA4.

15. The method of claim 14, wherein the soluble form of CTLA4 is a human CTLA4-immunoglobulin fusion protein.

16. The method of claim 12, wherein the agent is an anti-B7-1 antibody, or fragment thereof.

17. The method of claim 12, wherein the agent is an anti-B7-2 antibody, or fragment thereof.

18. The method of claim 12, wherein the agent is an anti-B7-1 antibody and an anti-B7-2 antibody, or fragments thereof.

19. The method of claim 12, further comprising administering the first population of cells to the recipient.

20. The method of claim 19, further comprising administering the agent to the recipient.

FIG. 1

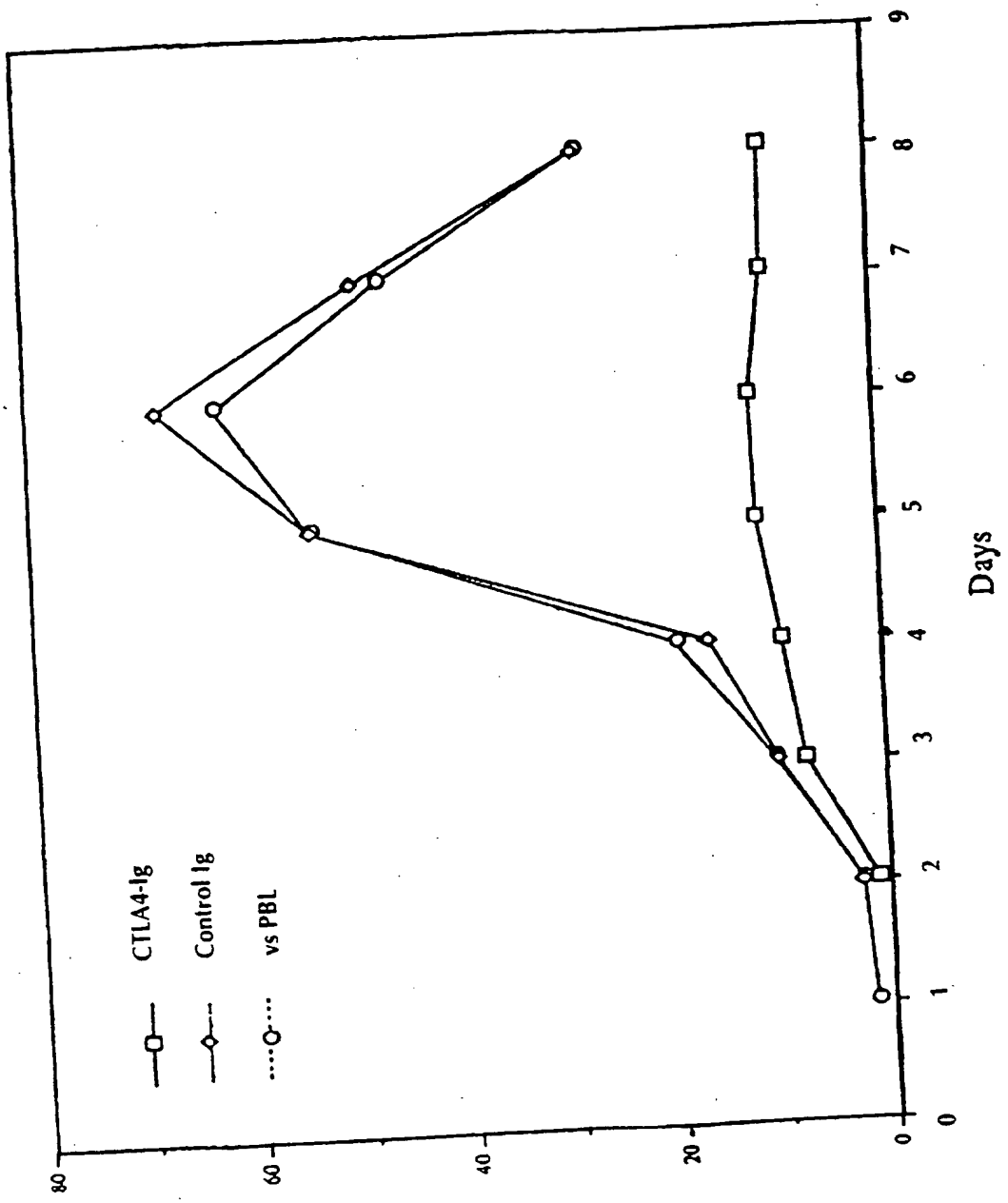
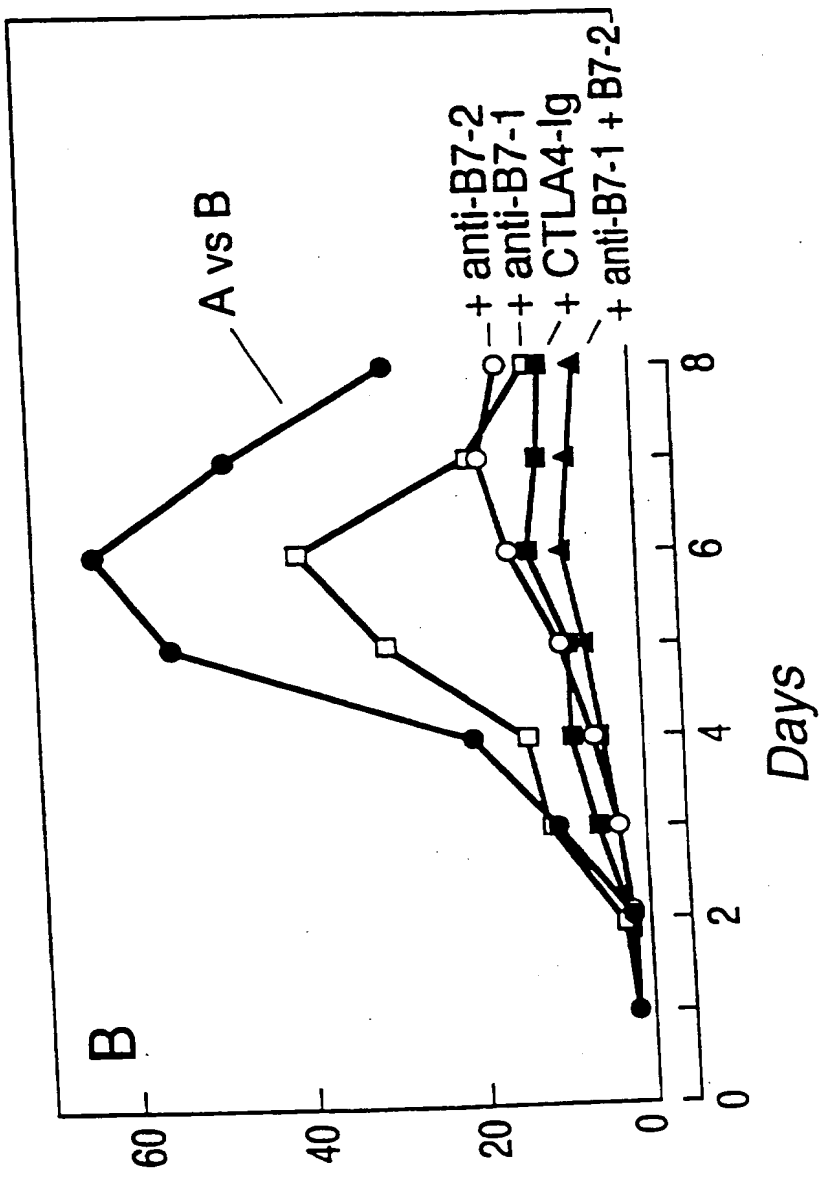

CTLA4-Ig
Control Ig
vs PBL
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
Days
Thymidine Incorporation (cpm x 10-3)

FIG. 2



[3H] THYMIDINE INCORPORATION
(cpm x 10^{-3})

FIG. 3A

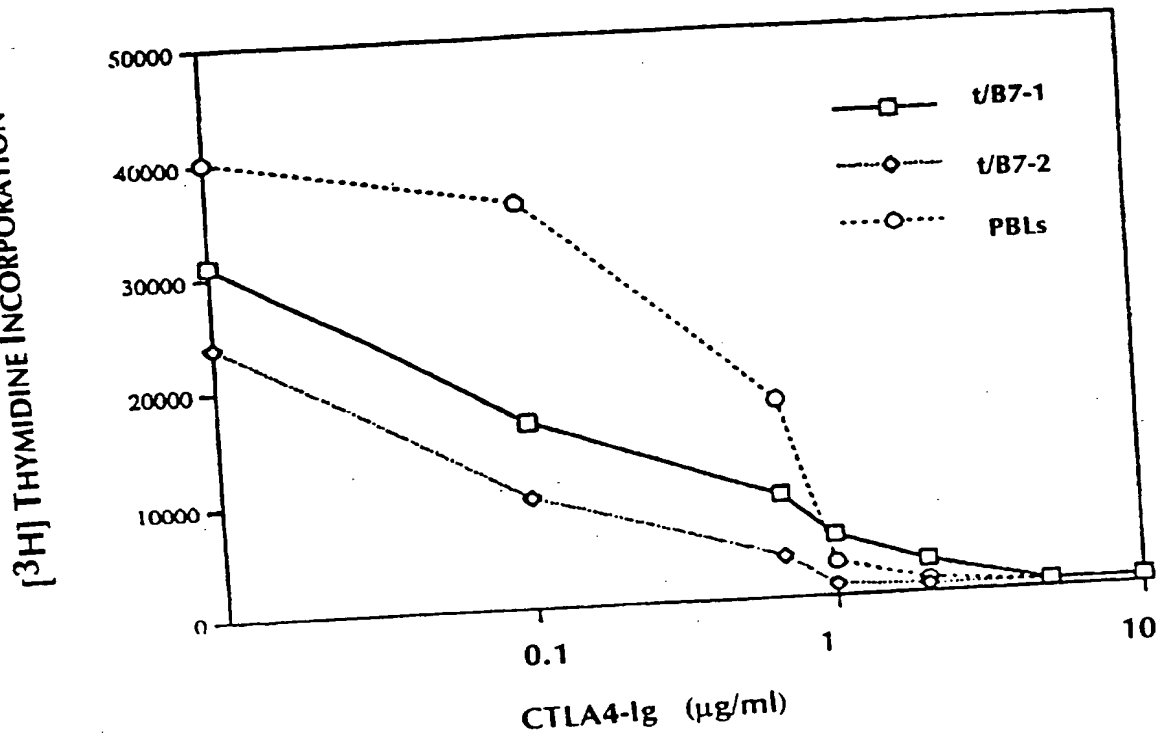

[3H] THYMIDINE INCORPORATION
50000
40000
30000
20000
10000
0
0.1
1
10
CTLA4-Ig (μg/ml)
t/B7-1
t/B7-2
PBLs

FIG. 3B

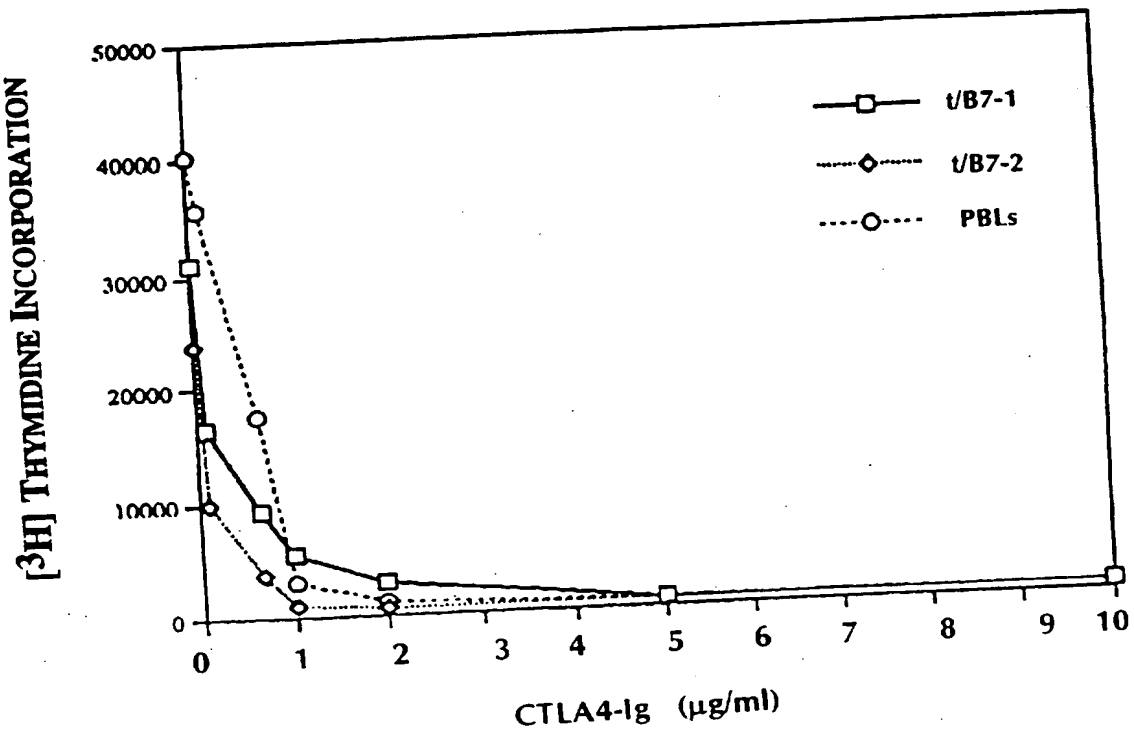

[3H] Thymidine Incorporation
50000
40000
30000
20000
10000
0
0
1
2
3
4
5
6
7
8
9
10
CTLA4-Ig (μg/ml)
t/B7-1
t/B7-2
PBLs

FIG. 4

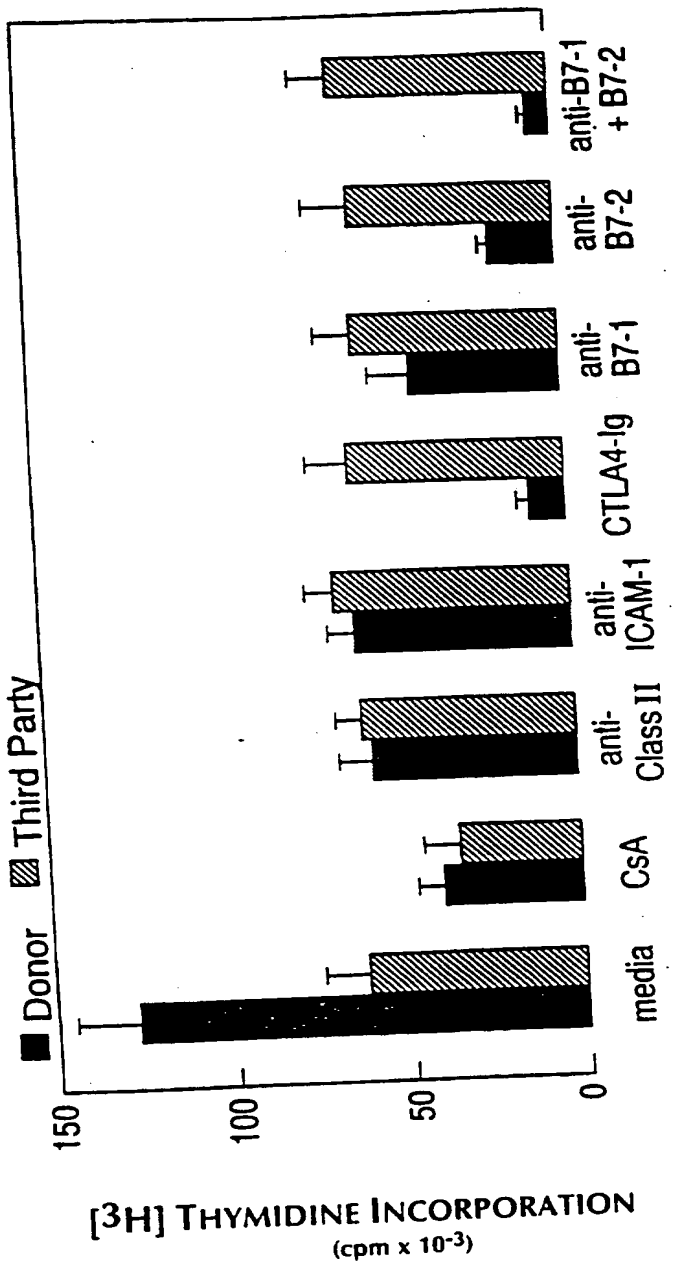

Donor
Third Party
media
CsA
anti-Class II
anti-ICAM-1
CTLA4-Ig
anti-B7-1
anti-B7-2
anti-B7-1 + B7-2
150
100
50
0
[3H] THYMIDINE INCORPORATION
(cpm x 10-3)

FIG. 5

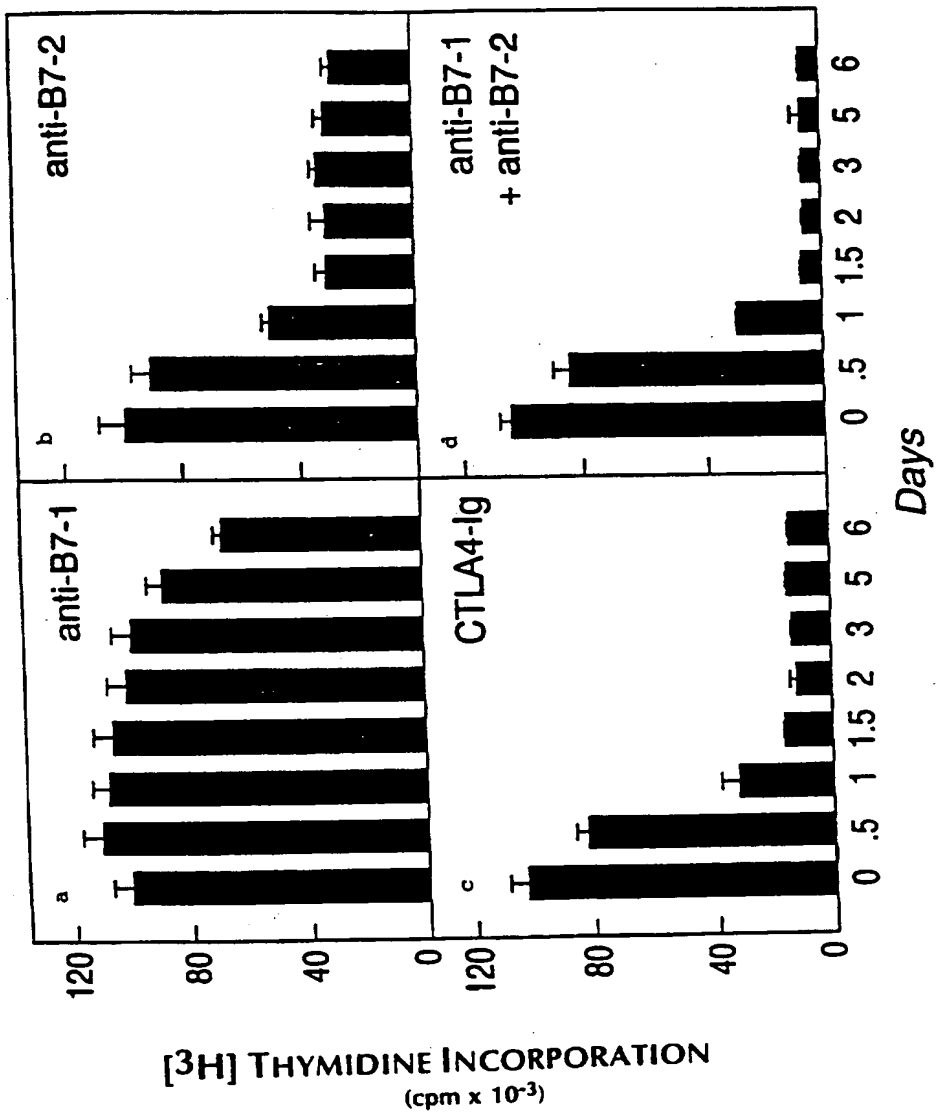

anti-B7-2
anti-B7-1
anti-B7-1
+ anti-B7-2
CTLA4-Ig
a
b
c
d
120
80
40
0
0 .5 1 1.5 2 3 5 6
Days
[3H] THYMIDINE INCORPORATION
(cpm x 10-3)

FIG. 6

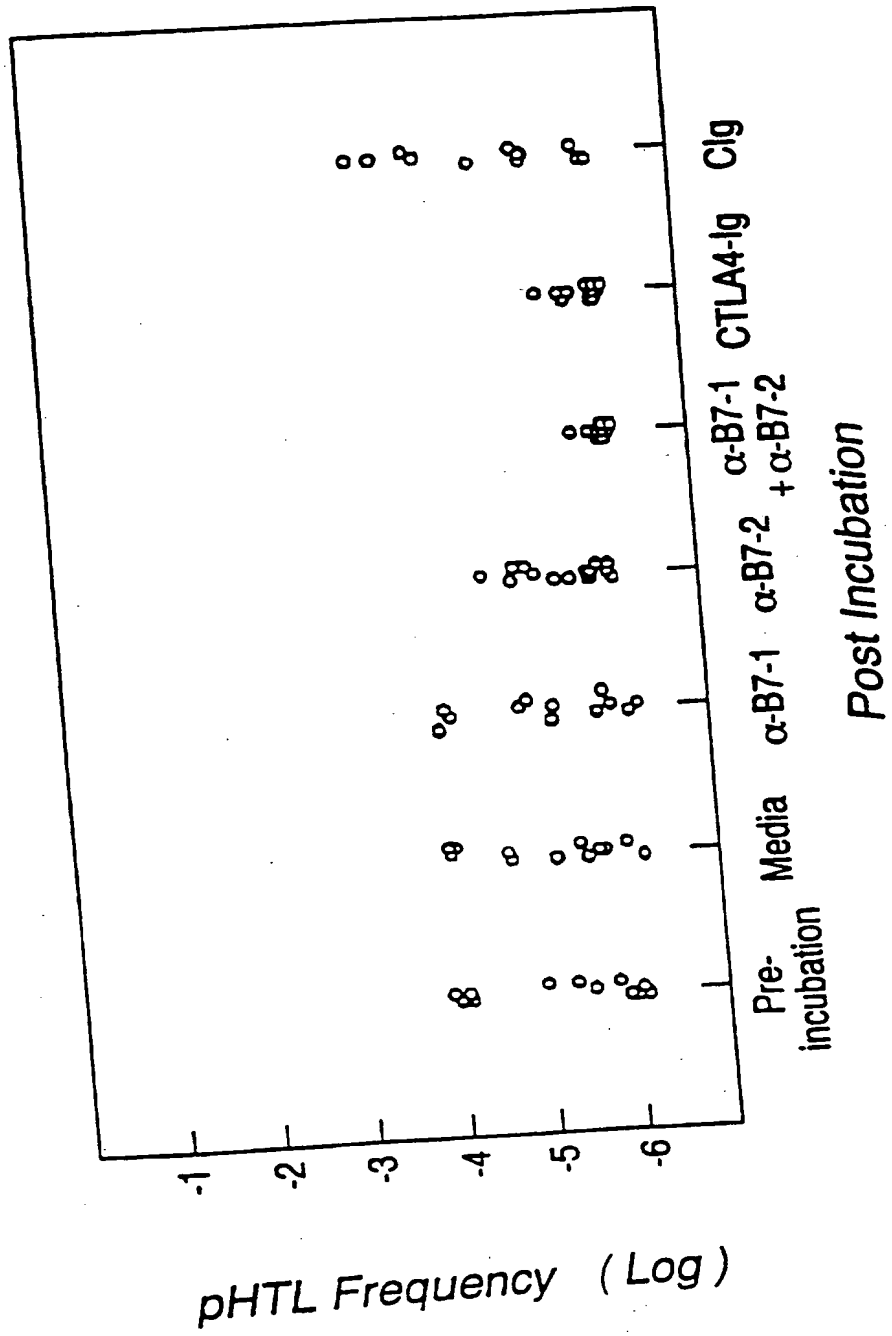

Pre-incubation
Media
α-B7-1
α-B7-2
α-B7-1 +α-B7-2
CTLA4-Ig
Clg
Post Incubation
-1
-2
-3
-4
-5
-6
pHTL Frequency (Log)

FIG. 7

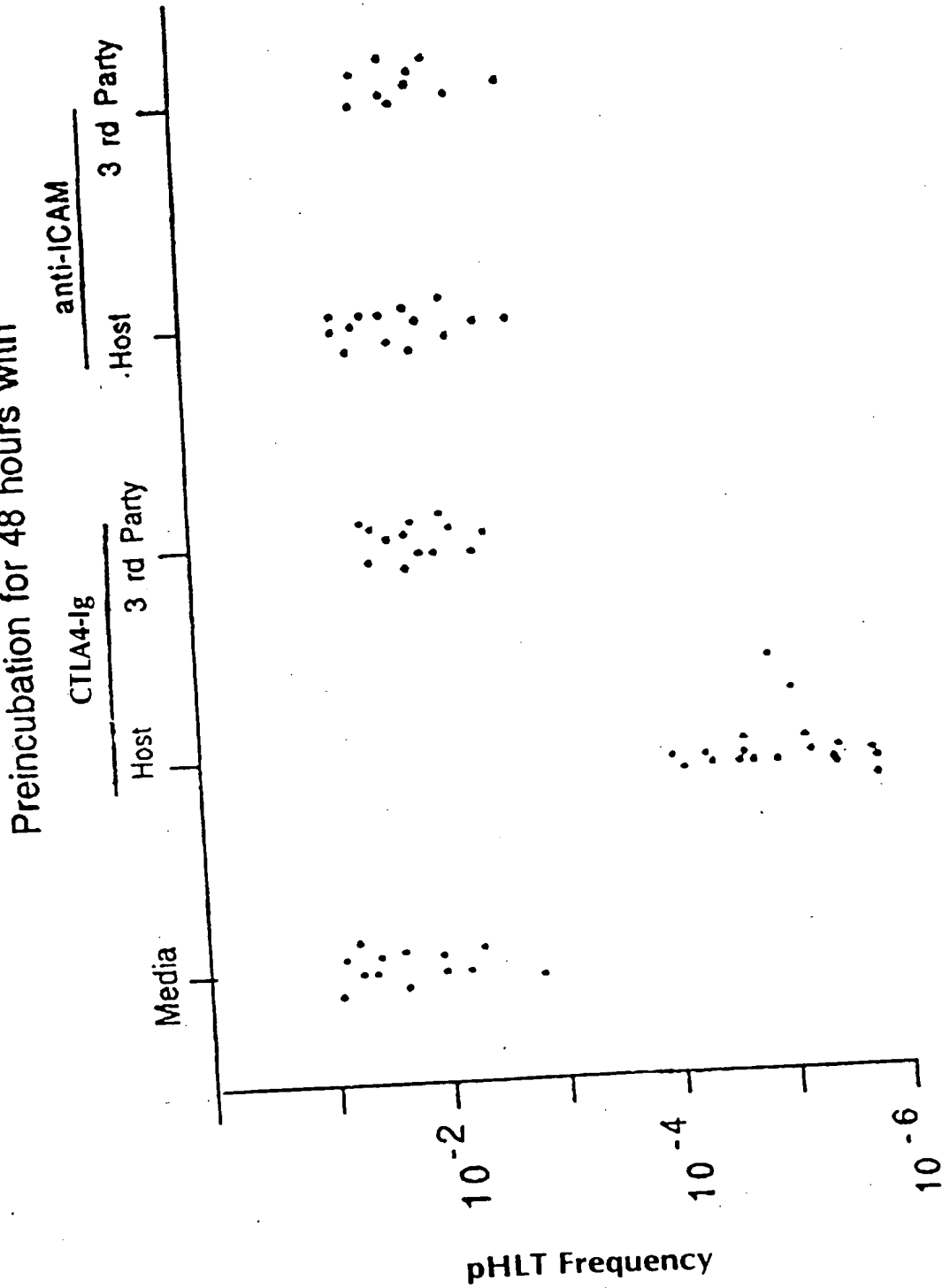

Preincubation for 48 hours with
Media
CTLA4-Ig
Host
3 rd Party
anti-ICAM
Host
3 rd Party
10^{-2}
10^{-4}
10^{-6}
pHLT Frequency

FIG. 8

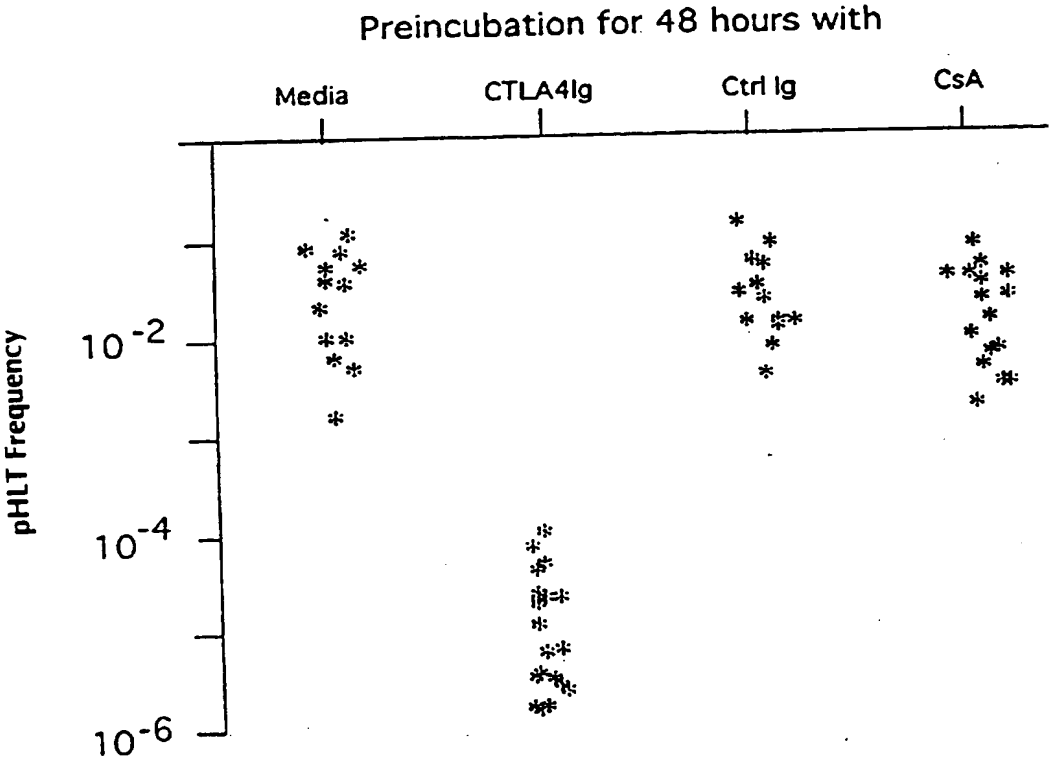
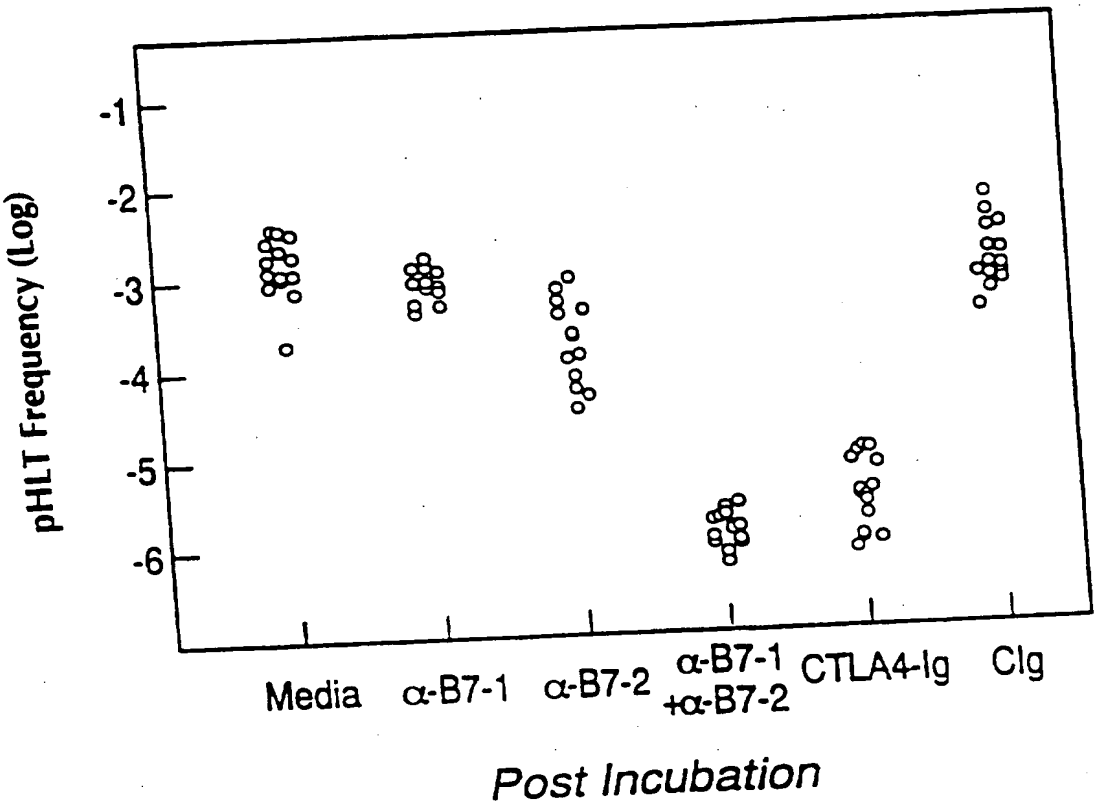

Preincubation for 48 hours with
Media
CTLA4Ig
Ctrl Ig
CsA
pHLT Frequency
10^{-2}
10^{-4}
10^{-6}

FIG. 9


pHLT Frequency (Log)
-1
-2
-3
-4
-5
-6
Media
α-B7-1
α-B7-2
α-B7-1 +α-B7-2
CTLA4-Ig
Clg
Post Incubation

INTERNATI L SEARCH REPORT

Interr [redacted] lication No
PCT/US [redacted] 5/14774

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/00 C07K16/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,95 03408 (DANA FARBER CANCER INST INC ;REPLIGEN CORP (US)) 2 February 1995 see page 6, last paragraph see claim 142 et seq. ---	1-19
P,X	THE JOURNAL OF IMMUNOLOGY, vol. 155, no. 8, October 1995 pages 3856-3865, PAUL G. SCHLEGEL ET AL. 'Inhibition of T Cell Costimulation by VCAM-1 Prevents Murine Graft-Versus-Host Disease Across Minor Histocompatibility Barriers' see page 3863, right column, last paragraph --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 April 1996	16.04.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Halle, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PERSPECTIVES IN DRUG AND DISCOVERY DESIGN, vol. 2, no. 1, 1994 pages 221-231, PETER S. LINSLEY ET AL. 'Immunosuppression and the CD28 receptor' see Bone marrow transplantation, pages 228-229 ---	1-19
Y	BLOOD, vol. 82, no. 10 Suppl. 1, 1993 page 456A B. R. BLAZAR ET AL. 'In vivo infusion of soluble CTLA4-Ig reduces lethal graft-versus-host disease (GVHD) induced across the major histocompatibility complex (MHC) barrier in mice' see the last sentence ---	1-19
E	WO,A,95 34320 (UNIV MINNESOTA) 21 December 1995 see page 2, Summary of the invention -----	

1

INTERNATIONAL SEARCH REPORT

information on patent family members

Intern. Application No PCT/US95/14774

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9503408	02-02-95	AU-B- 7405294	20-02-95
WO-A-9534320	21-12-95	AU-B- 2701895	05-01-96